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14. **ABSTRACT**
Perlecan (Pln), the major heparan sulfate proteoglycan (HSPG) in the bone marrow stromal ECM functions as a permissive ECM molecule, or co-receptor, that delivers HBGFs to high affinity receptors. Heparan sulfate chains on Pln are located in a unique N-terminal domain I (PlnDI) which functions as the co-receptor for HBGFs. Our purpose is to study the functions of Pln in prostate cancer cell survival, proliferation and apoptosis. We hypothesize that Pln, acting through PlnDI, delivers HBGFs to the cancer cell surface, promotes proliferation, cell survival and protects them from apoptosis. The Pln knockdown studies showed that Pln knockdown clones grow poorly compared to the parental or control transfected C4-2B cells. PlnDI failed to rescue the poorly proliferation. Western blot of Akt/MAPK pathway of these knockdown clones showed that Pln is not a major regulator in these pathways. We used three different prostate cancer cell lines: LnCaP, its derivative, C4-2B, and PC3 as model systems to study the protection function of PlnDI. We harvested conditioned medium from bone stromal cell lines, HS27a and HS5, which produce abundant Pln. The proteoglycan rich fraction containing Pln was obtained using anion exchange bead chromatography, then tested as a survival factor for the three different prostate cancer cell lines. DNA fragmentation experiments showed that bone stromal derived Pln from conditioned medium protected all three lines of prostate cancer cells from camptothecin induced apoptosis. We also supplied purified, fully glycosylated recombinant PlnDI protein to prostate cancer cells, which also protected cells from apoptosis. The Pln knockdown cells are also more susceptible in response to the apoptosis inducers anti-Fas and camptothecin, and that exogenous PlnDI can rescue survival. Taken together, these studies demonstrate that Pln present in the bone marrow compartment can promote cell proliferation. It can also protect prostate cancer cells from apoptosis, and that this activity maps to heparan sulfate bearing PlnDI.

15. SUBJECT TERMS
Pelecan, HBGFs, Prostate Cancer, apoptosis

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Introduction

Pln domain I (PlnDI) is a unique module at the N terminus of Pln and contains three closely spaced Ser-Gly-Asp sequences that serve in GAG attachment [1]. PlnDI with three HS chains can bind a number of matrix molecules, cytokines, and growth factors [2]. Our previous studies indicate that the GAG-bearing PlnDI provides a sufficient signal to trigger C3H10T1/2 cells to enter a chondrogenic differentiation pathway and the GAGs are important for this process because PlnDI-based polypeptides lacking GAG chains either by enzymatic removal or mutation of HS/CS attachment sites were inactive [3]. It is precisely this growth factor delivery "co-receptor" function of PlnDI that we have begun to explore with regard to prostate cancer bone metastasis and growth.

During the first year of the training period, I focused on the role of PlnDI in cell proliferation, cell survival and apoptosis by using different prostate cell lines and Pln knock down clones. The statement of work and planned goals have not changed significantly from those described in the original proposal, and significant progress has been made toward the aims (see below).

Main Body

Specific Aim1: To test the co-receptor function of PlnDI in prostate cancer cell proliferation.

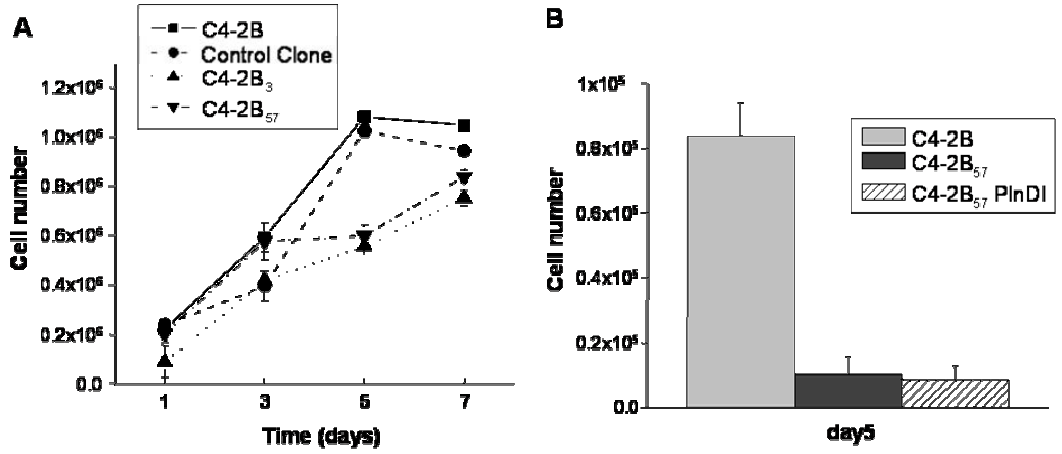

Figure 1. Addition of exogenous Pln does not rescue growth responses to FGF-2 in Pln knockdown C4-2B cells. Panel (A). Conditioned medium from parental C4-2B cells failed to rescue the proliferation of Pln knockdown clones C4-2B$_3$ and C4-2B$_{57}$ cultured in FGF-2. Panel (B). Purified Pln domain I protein (5 ug/ml) failed to rescue the growth response to FGF-2, whereas parental cells continued to proliferate through the fifth day in culture. Figure is adapted from [4].

Preliminary experiments tested the HB growth factor dependent responses of Pln knockdown clones and compared them with their parental cell line C4-2B or control transfectants [4]. We used a structure-function based approach to determine if PlnDI, specifically the GAG chains and/or the core protein in domain I, are sufficient to provide

this co-receptor function. Surprisingly, although Pln knockdown clones have lower proliferation rates, adding purified PlnDI in the medium failed to rescue the phenotype. Because the intact PlnDI didn't rescue the reduced proliferation, we didn't conduct the GAG chain study (Fig. 1). Our hypothesis is that PlnDI only is not sufficient for the co-receptor function involved in proliferation. This study was included in one of our publication. (Appendix 1)

Specific Aim2: To test if PlnDI is involved in activating the cell survival pathway.
We measured activation of the cell survival pathway Akt and MAPK by western blot and compare responses of the Pln knockdown clones and the parental C4-2B cell line. Although the proliferation rate of the knockdown clones was different, there were no detectable changes in Akt or MAPK pathways of these knockdown clones (Fig. 2). Thus, we didn't continue to study the role of PlnDI involved in these pathways. The key factors in these pathways are highly expressed and constitutively active in these prostate cancer cell lines. This could be one explanation why we failed to detect moderate changes due to Pln knockdown. Another hypothesis is that Pln regulated proliferation goes through other pathways, but not Akt/MAPK.

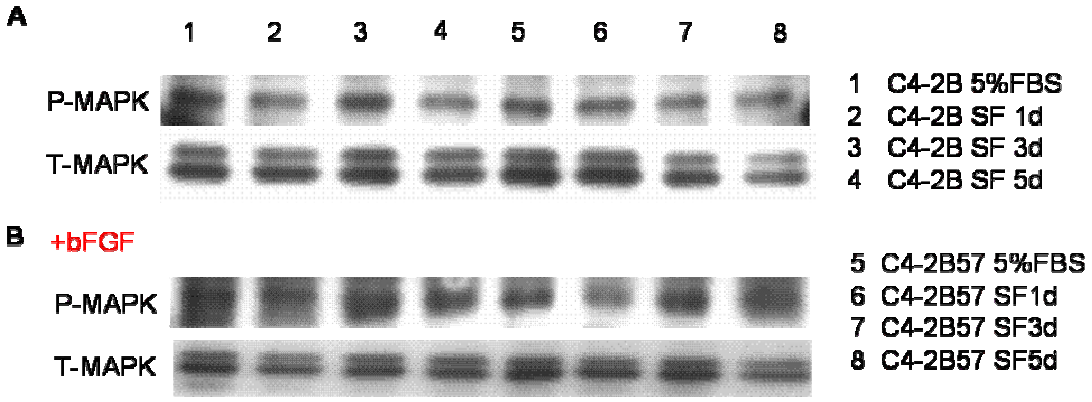

Figure 2. Western blots showed that phosphorylated MAPK levels were not altered but constitutively on in the Pln knockdown clone 57. P-MAPK: phosphorylated MAPK. T-MAPK: Total MAPK.

Specific Aim3: To test if PlnDI protects prostate cancer cells from apoptosis.
To study the role of PlnDI involved in apoptosis, we expanded the study to three prostate cancer cell lines: LNCaP, PC3 and C42B in addition to the knock down clones. We used two bone stromal cell lines HS27a and HS5 to harvest conditioned medium. The proteoglycan rich fraction containing Pln was obtained using anion exchange bead chromatography. Applying the Pln enriched medium to all three prostate cancer cell lines showed less apoptosis compare to serum free medium when treated with camptothecin (Fig. 3). This experiment indicated that the Pln in the bone environment might protect prostate cancer cells from apoptosis. We also applied purified PlnDI protein to these cells and we successfully rescued the apoptosis (Fig.4). Then we measured the ability of PlnDI added exogenously to Pln knockdown clones to protect them from apoptosis induced by

anti-Fas or camptothecin. We showed that PlnDI can rescue the increased apoptosis caused by Pln knockdown (Fig. 5). <u>These data suggest that Pln can protect prostate cancer cell from apoptosis and PlnDI is sufficient for this function.</u> We plan to expand our study by using Pln antibody depletion to ensure the role of Pln in cell apoptosis.

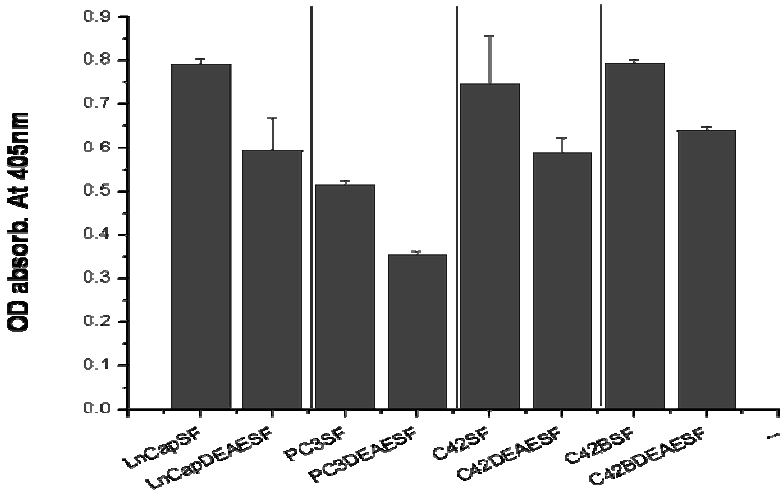

Figure 3. Pln enriched medium reduced apoptosis in all cell lines treated with camptothecin. SF: Serum free medium. DEAESF: DEAE treated conditioned serum free medium from bone stromal cells which contains enriched proteoglycan (Pln).

The only original aim that we have not yet performed is to dissect the structure-function aspects of Pln in prostate cancer behavior. We will study PlnDI versus intact Pln, and then determine if the GAG portion and/or the core protein of PlnDI are required. Finally, heparitinase and chondroitinase digestion, and GAG rescue experiments will be performed. <u>By performing these structure-function analyses, we will be able to test the function of PlnDI, the individual GAGs and the core protein in controlling prostate cancer apoptosis.</u>

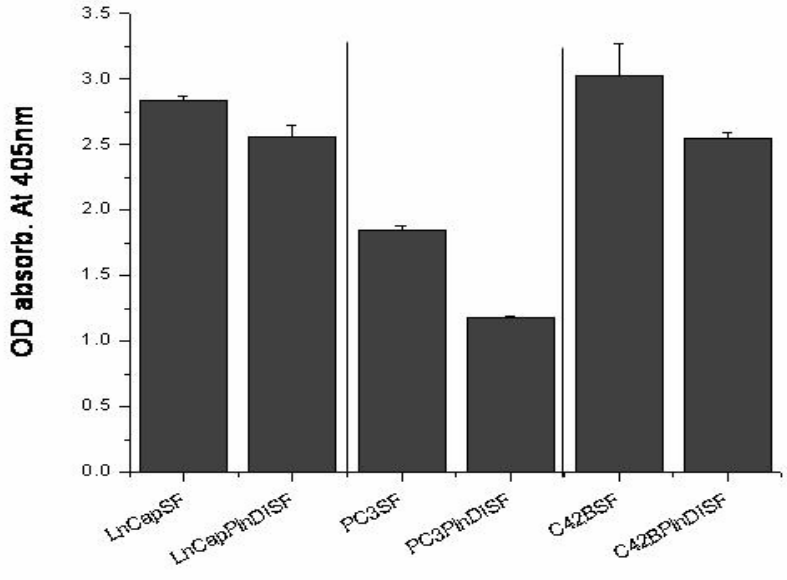

Figure 4. PlnDI reduced apoptosis in all cell lines treated with camptothecin. SF: Serium free medium. PlnDISF: SF containing PlnDI protein.

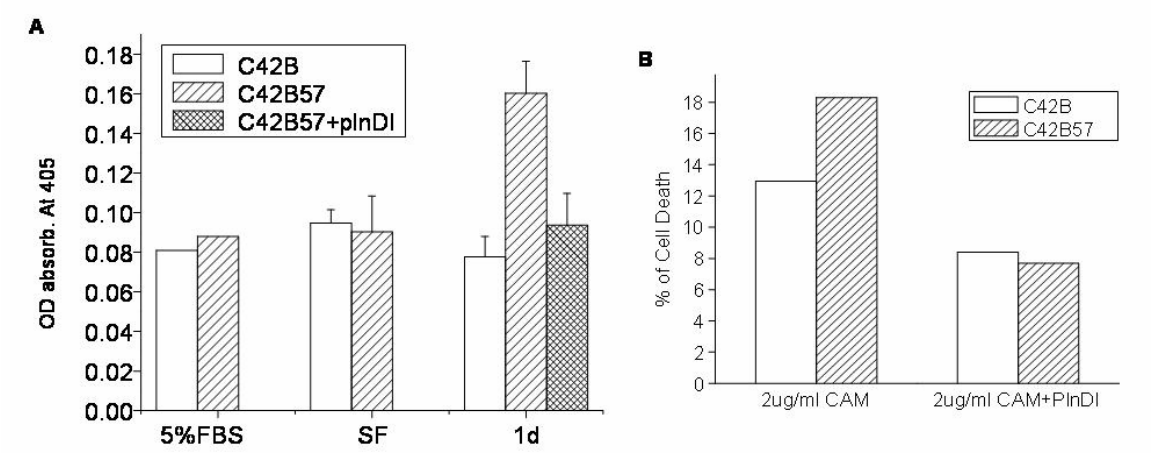

Figure 5. Pln knockdown clones had more apoptosis compare to parental cells and PlnDI could rescue apoptosis induced by anti-Fas or camptothecin. Panel (A). DNA fragmentation assay showed that Pln knockdown clone had more cell death in response to anti-Fas treatment. Panel (B). Pln knockdown clone had more cell death in response to camptothecin.

Key Research Accomplishments:

We have achieved many research accomplishments emanating from the training grant:

- We successfully showed that PlnDI itself is not sufficient for cell proliferation.
- We studied cellular pathways involved in cell survival and showed that the Akt/MAPK pathway is not regulated by PlnDI.
- We successfully rescued prostate cancer cells form apoptosis by Pln enriched conditioned medium from bone stromal cells.

Reportable Outcomes

- **Poster presentations at international conference:**
  Function of Perlecan in Prostate Cancer Cell Growth and Survival. 8th International Conference on the Chemistry & Biology of Mineralized Tissues, October 17 -22, 2004, Banff, Canada

Conclusions

During the first year supported by this Training Grant, we successfully completed more than two thirds of our proposed experimental aims:

- **Pln and cell proliferation:** From Pln knockdown studies, we showed that Pln regulates prostate cancer cell proliferation but PlnDI alone is not sufficient for this function.
- **Pln and cell survival:** We showed that Akt/MAPK pathways are not critical in Pln regulated cell proliferation.
- **Pln and cell apoptosis:** We studied apoptosis of different prostate cancer cell lines in response to apoptosis inducer camptothecin. We successfully rescued apoptosis of all prostate cancer cell lines by Pln enriched conditioned medium from bone stromal cells. We also showed that PlnDI has the same rescue effect as

Pln enriched conditioned medium. We further studied apoptosis of the Pln ribozyme knockdown clones induced by anti-Fas or camptothecin and when compared to parentals, the knockdown clones showed more apoptosis. We showed that purified PlnDI protein can rescue the apoptotic behavior of the knockdown clones to more closely resemble the parentals that express normal levels of Pln.

- **Future work:** Our next step is to further test if the GAGs are essential for the function of PlnDI by attempting rescue with HS or CS. We also will perform selective enzyme digestions with chondroitinase or heparinase to measure the relative contributions of the GAGs and the core protein.
- **Prospectus:** These systematic studies will dissect the role of PlnDI in controlling prostate cancer cell survival, proliferation and apoptosis.

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Appendices

Appendix 1 See attached PDF paper

Clinical & Experimental Metastasis (2005) 22: 377–390
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Perlecan knockdown in metastatic prostate cancer cells reduces heparin-binding growth factor responses *in vitro* and tumor growth *in vivo*

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Key words: angiogenesis, extracellular matrix, heparin binding growth factors, perlecan, prostate cancer, ribozyme

Abstract

Perlecan (Pln) is a major heparan sulfate proteoglycan (HSPG) of extracellular matrices and bone marrow stroma. Pln, via glycosaminoglycans in domains I and V, acts as a co-receptor for delivery of heparin binding growth factors (HBGFs) that support cancer growth and vascularization. Specifically, glycosaminoglycans bind HBGFs and activate HBGF receptors, including those for FGF-2 and VEGF-A. The contribution of Pln to prostate cancer growth was tested using a ribozyme approach to knockdown Pln expression levels. Transfection into the androgen-independent, bone targeted prostate cancer line, C4-2B, and efficient stable knockdown of Pln was demonstrated by quantitative PCR, immunohistochemistry and immunoblotting. Three individually isolated subclones with 75–80% knockdown in Pln mRNA, protein expression and secretion into ECM were used to study *in vitro* growth responses to FGF-2 and VEGF-A. While cells with normal Pln levels responded to both HBGFs, knockdown cells responded poorly. All lines responded to serum growth factors and IGF-I. Anchorage-independent growth assays showed reduced colony size and cohesiveness by all Pln deficient subclones compared to parental C4-2B cells. *In vivo* effects of Pln knockdown were measured by inoculating knockdown and control ribozyme transfected cell lines into athymic mice. A reduced growth rate, smaller tumor size, diminished vascularization and failure to elevate serum PSA characterized mice bearing Pln knockdown C4-2B cells. Poor vascularization correlated with reduced levels of VEGF-A secreted by Pln knockdown lines. We conclude that Pln is an essential ECM component involved in growth responses of metastatic prostate cancer cells to HBGFs deposited in local and metastatic microenvironment.

Abbreviations: ECM – extracellular matrix; FBS – fetal bovine serum; FGF-2 – basic fibroblast growth factor; FGF-BP – fibroblast growth factor binding protein; HBGF(R) – heparin binding growth factor (receptor); HSPG – heparan sulfate proteoglycan; PBS – phosphate buffered saline; Pln – perlecan; SCID – severe combined immunodeficient; VEGF-A – vascular endothelial growth factor

Introduction

The extracellular matrix (ECM) provides a dynamic microenvironment surrounding cancer cells that contributes to growth and phenotypic transformation during tumorigenesis by acting at both cellular and molecular levels [1]. Understanding the mechanisms regulating the interactions between cancer cells and ECM thus creates new paradigms by which targeted therapeutic interventions for this deadly disease can be developed. In normal tissues, a precise and coordinated network of interactions exists that involves heparan sulfate proteoglycans (HSPGs), heparin binding growth factors (HBGFs) and heparin binding growth factor receptors (HBGFRs). These three components provide a mechanism for maintenance of homeostasis and vascularization during tissue growth and remodeling.

In prostate cancer, a relatively slow-growing primary tumor forms in the prostate which, if untreated, can develop into a metastatic disease that predictably forms highly metastatic secondary tumors in bone. The arrival of metastatic prostate cancer cells to the bone stroma disrupts the pre-existing homeostasis in this environment and supports the growth and progression of osteoblastic metastases. HBGFs produced by bone

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marrow and by prostate cancer cells themselves are sequestered in the bone ECM where they support growth, survival and motility of metastatic prostate cancer cells. HBGFRs depend on HSPGs as co-receptors, which form complexes with HBGFs to protect them from proteolysis in the tumor microenvironment [2]. Perlecan (Pln), a large secreted HSPG, is a major component of basement membranes, bone stroma and blood vessels that support high affinity growth factor binding by nearby receptors [3, 4]. It is the major HSPG present in the bone marrow stromal ECM [4] and recently was shown to support tumor progression and vascularization [5] in colon carcinoma. Increased Pln levels were demonstrated in breast carcinomas [6], and in metastatic melanomas [7] and correlated with a more aggressive phenotype [8]. Recent work demonstrated that the knockdown of FGF-BP, a novel ligand for Pln protein core, reduces tumor growth rates *in vivo* and *in vitro* [9]. Previous studies showed that antisense targeting of Pln blocks colon carcinoma growth and angiogenesis *in vivo* [5], and a separate study showed that loss of Pln has complex effects on growth of knockdown clones of Kaposi sarcoma cells [10]. In our current study, we used an active ribozyme targeting Pln [11] to determine the role it plays in HBGF stimulation of prostate cancer cell growth and survival under various conditions *in vitro*. Pln expression was knocked down in C4-2B cells, a human prostate cancer cell line with high metastatic potential and increased osteomimetic differentiation [1, 12]. Several criteria were used to assess the nature and extent of the growth responses to HBGF of the Pln deficient subclones *in vitro* in cell culture and *in vivo* using an immune-deficient mouse model. Our results indicate that Pln is essential for cellular responses to HBGFs, including FGF-2, which is abundantly produced in the bone marrow environment, and vascular endothelial growth factor-A (VEGF-A), the major HBGF produced by C4-2B cells (Muir et al., in preparation). Moreover, we show that Pln biosynthesis is necessary for anchorage-independent growth under a variety of *in vitro* conditions and for tumor growth and vascularization in a SCID mouse model.

Materials and methods

Cell lines

PC3 cells and LNCaP cells were maintained as described previously [13, 14]. Stocks of the cell line C4-2B (subline 4) were maintained in T medium (Invitrogen, Carlsbad, CA) containing 10% (v/v) fetal bovine serum (FBS). Ribozyme transfected clones were maintained in the T medium containing 10% (v/v) FBS, supplemented with 225 μg/ml of Zeocin (Invitrogen). At the time of the experiments, the cells were transferred into DMEM low glucose (Invitrogen).

Ribozyme production

The ribozyme targeting Pln transcript was produced exactly as recently described in a manuscript using the production of the Pln ribozyme to illustrate our strategy and *in vitro* testing procedures [11]. Briefly, target substrate RNA and two candidate ribozymes were synthesized and analyzed *in vitro* as described. The efficiency of the ribozyme cleavage reactions was measured and the ribozyme determined to be most efficient (ribozyme 396) was selected for transfection into C4-2B cells. This ribozyme targets a sequence that translates into the midportion of the sequence encoding the SEA module of domain I of Pln (http://www.sanger.ac.uk/Software/Pfam/). Additionally, a control ribozyme with scrambled annealing arm sequences also was synthesized. Active ribozyme 396 and control ribozyme were cloned into the modified plasmid pZeoU1EcoSpe as we described previously [15]. The sequence and orientation of the inserts as well as the U1 snRNA expression cassette were confirmed by DNA sequencing.

Production of knockdown clones

C4-2B cells were plated into 24 well plates (Corning Incorporated, Acton, MA) at a density of 1×10^4 cells/well and allowed to attach for 24 h in 10% (v/v) FBS containing DMEM low glucose (Invitrogen). Transfections were performed using LipofectAMINE™ Reagent (Invitrogen), following the manufacturer's instructions. Briefly, when judged by eye to reach 70–80% confluency, the cells were incubated overnight at 37 °C in 5% (v/v) CO_2 with the transfection mixture containing 2 μg of DNA (ribozyme 396 plasmid cDNA encodes a Pln ribozyme) and 3 μl of LipofectAMINE™ Reagent in 100 μl of medium without FBS. After incubation, the mixture was removed and substituted with complete growth medium for 48 h. After 72 h of transfection, the cells were passaged in the selection medium containing 300 μg/ml Zeocin. After 12 to 14 days, colonies were isolated following a published protocol [16], using a 200 μl tip, plated individually into 6-well plates in DMEM containing 225 μg/ml Zeocin and the colonies formed were expanded subsequently in 10% (v/v) FBS containing T medium (Invitrogen). Immediately after isolation and propagation of the stable clones, the knockdown subclones were frozen down and stored in liquid nitrogen in aliquots. Subclone stocks were maintained in 10% (v/v) FBS containing T medium (Invitrogen) supplemented with 225 μg/ml Zeocin and used for experiments for a maximum of 10 passages. Cells were withdrawn from Zeocin for one passage in DMEM prior to use in individual experiments that were performed in the absence of the selection antibiotic.

RT-PCR

RNA was extracted from all three cell lines using the RNeasy™ Kit (Qiagen, Valencia, CA) and treated with

DNase (Ambion, Austin, TX). cDNA was synthesized from total RNA using the 1st strand cDNA synthesis kit from BD Biosciences/Clontech (Palo Alto, CA). RT-PCR was performed as described previously [15]. The amplification region includes an approximately 1100 bp segment comprising most of domain I and a portion of domain II, near the 5′ end of the transcript where the ribozyme targeted sequences are located. Each reaction mixture contained cDNA synthesized from 3.1 μg of total RNA. The following primers were used for detection of Pln by RT-PCR:

HPLNF :5′-GAGGGCATACGATGGCTTGT-3′,
HPLNR: 5′-AGTGGAAGCTGGCTGGGAT-3′.

Real time PCR

RNA was extracted from the clones and treated with DNase as described above. One hundred ng of RNA were reverse transcribed using Omniscript™ RT Kit (Qiagen) and random hexamers (Applied Biosystems Foster City, CA). RT-PCR was performed using Quantitect™ SYBR Green Kit (Qiagen). The following primers for the real time reactions were selected based on the domain V sequence of Pln and GAPDH was used as control:

HPLNVF: 5′-ACCATCGAGCTGGAGGTTC-3′,
HPLNVR: 5′-GAGGCTGATGAAGTCCTTGC-3′,
(151 bp).
GAPDHF: 5′-GCTGAGTATGTCGTGGAGTC-3′,
GAPDHR: 5′-TTGGTGGTGCAGGATGCATT-3′
(191 bp).

Standards were generated by cloning the sequence verified PCR product into the pCR2.1 vector using TOPO T/A cloning kit from Invitrogen. Isolated plasmid was linearized using *EcoRV*, quantitated and diluted to use as standards in real-time RT-PCR reactions. Real Time PCR was performed in the iCycler iQ™ Real Time PCR detection system from Bio-Rad Laboratories (Hercules, CA). After 10 min incubation at 95 °C, the cycling conditions were as follows: denature at 94 °C for 60 sec, annealing at 58 °C for 50 sec, and extension for 60 sec at 72 °C for 45 cycles. All determinations reported are based on 3–9 replicates. Data was analyzed on Prism 3.0 from GraphPad Prism® and error bars reflect determinations of standard error (San Diego, CA).

Immunohistochemical localization studies

Cells were plated in chambered coverslips (Lab-tek® Products, Nalge Nunc International, Naperville, IL) in 10% (v/v) FBS DMEM low glucose medium until they reached 70% confluency. The medium then was removed and cells were washed twice with PBS and fixed in cold methanol on ice for 10 min. The methanol was removed and the cells were rehydrated with PBS (3 times for 5 min each), after which the cells were blocked in 3% (w/v) BSA in PBS for 1 h at room temperature, followed by incubation with a well characterized rabbit polyclonal antibody [17] to Pln (generous gift from Dr J. Hassell, Shriners' Hospital, Tampa Bay, FL) at a dilution of 1:200 for 1 h at 37 °C in a humidified chamber. The solution containing the primary antibody was removed and the cells were washed 3 times with PBS. The cells were incubated with the secondary antibody (FITC conjugated anti-rabbit, Jackson Immunochemical Research West Grove, PA) diluted 1:200 for 1 h at 37 °C in a humidified chamber. The cells were washed with PBS and observed immediately using a multiphoton confocal microscope (Leica, Jena, Germany).

Immunohistochemical studies of neovasculature of tumor specimens was conducted using modifications of previously published methods [18–20] except that a Dako Autostainer Plus system (Dako Corporation, Carpinteria, CA) was used. Tissues were deparaffinized, rehydrated and pressure-cooked for 5-min-antigen retrieval. Mouse anti-human endothelial cell monoclonal antibody, CD31, clone JC70A, was used in conjunction with DakoCytomation EnVision$^+$. A HRP detection system using diaminobenzidine as chromogen and hematoxylin for counterstaining was employed. All reagents were obtained from Dako Corporation.

Dot-blot quantification of Pln protein levels

Cells were grown in 6-well plates in 10% (v/v) FBS DMEM low glucose medium until they reached approximately 90% confluency. The medium then was substituted with FBS-depleted medium. After 48 h, medium was collected and cleared of particulate material using a microcentrifuge at 13.2 × g for 20 min at 4 °C. Using a vacuum manifold 100 μl of the medium was loaded onto a nitrocellulose membrane. Serum depleted (0.2%) medium was loaded as control and a standard curve was prepared in parallel using serial dilutions of a known concentration (initial spot contained 20 ng) of pure Pln protein (generous gift of Becton and Dickinson, Franklin Lakes, NJ). All samples were analyzed in triplicate. Dot blot hybridization was performed using the same well characterized rabbit polyclonal antibody to Pln at a dilution of 1:200. The species-specific horseradish peroxidase conjugated secondary antibody was purchased from Amersham Corporation (Arlington Heights, IL).

Soft agar assay

The three Pln deficient subclones were grown in soft agar to evaluate their ability to form colonies. A base layer of agar, containing 0.5% (w/v) agar (Invitrogen), DMEM (Invitrogen) with 10% (v/v) FBS was layered on a two chambered glass coverslip (Lab-Tek® II, Nalge Nunc International, Naperville, IL). In each case, 5000 cells were plated onto the base layer and then topped

with a layer of 0.33% (w/v) agar containing DMEM plus 10% (v/v) FBS. Cells were grown for 14 days and then stained with Syto13 (Molecular Probes, Eugene, OR) and visualized using a multiphoton confocal microscope (Leica, Jena, Germany).

Growth factor responses

Ribozyme transfected sublines referred to as C4-2B$_3$, C4-2B$_{11}$ and C4-2B$_{57}$, and control ribozyme transfected and untransfected parental C4-2B control were plated in triplicate into 12-well plates at a density of 1.5×10^4 cells/well and allowed to attach for 24 h in 10% (v/v) FBS in DMEM. Cells then were switched into 0.2% (v/v) FBS in DMEM for 48 h, after which treatment with b-FGF, VEGF-A or non-heparin binding IGF (R&D Systems, Minneapolis, MN) was initiated using 30 ng/ml growth factor in 0.2% (v/v) FBS in DMEM. Growth factor concentrations were chosen from results of a dose-response experiment conducted on the C4-2B parental line (data not shown). In parallel, the clones and the parental cell line, C4-2B, were grown in DMEM (low glucose) containing 10% (v/v) or 0.2% (v/v) FBS as controls. The treatments were continued for 7 days, changing the growth factor containing medium as well as the control medium every 48 h. Cells were counted manually using a hemocytometer at the beginning of the treatment (time day 0), at day 3 and at day 7. All counts were performed in triplicate and repeated in two independent sets of experiments. For rescue experiments, conditioned DMEM medium was harvested from parental C4-2B cells grown in serum depleted (0.2%) medium, mixed 1:1 with new DMEM medium and then added to cells in culture. Pln domain I was purified and characterized as described previously [21] and added to cultures at a final concentration of 5 μg/ml, a concentration with maximal bioactivity in other assays routinely performed in our laboratory. For presentation of growth data, some experiments were normalized to the cell number at seeding that was assigned a value of 1.0; in other cases, direct cell counts are reported. An unpaired Student t test was performed to assess statistical significance. Error bars reflect standard error determinations, and experiments were repeated two or three times with triplicate samples.

Measurement of HBGF levels in conditioned medium

Cells were grown to approximately 70% confluency as judged by visual inspection in 10% (v/v) FBS DMEM low glucose in a T75 flask before being switched to serum depleted medium for 24 h. The conditioned medium was collected, centrifuged at $13.2 \times g$ for 2 min at 4 °C to remove debris, and stored as aliquots at −20 °C. HBGF levels in conditioned medium were determined using Human Quantikine ELISA assays for VEGF-A and FGF-2 (R&D Systems). Conditioned medium was thawed on ice and used directly, or diluted according to the manufacturer's directions. Absorbance was measured at 450 nm using a Dynex plate reader. For analysis, a standard curve relating optical density to protein concentration was used to determine protein concentrations in conditioned medium.

Mouse model harboring human prostate tumor cells

All procedures were performed in full accordance with IACUC approved protocol for care and use of animals. In brief, tumors were induced in SCID mice (two to three tumors per mouse, two mice per study) by subcutaneous inoculation of C4-2B, control ribozyme or C4-2B$_{57}$ cells according to previously published methods with slight modification [18, 19]. Cells (2×10^6) were resuspended in 0.1 ml of T medium containing 5% (v/v) FBS, and a 1:1 dilution of Matrigel™ (1:1 dilution, BD Biosciences, Franklin Lakes, NJ), and then injected subcutaneously into the left flank of 6–8 week old male SCID/bg mice (National Cancer Institute, Bethesda, MD) using a tuberculin syringe with a 27 g needle. Two complete studies were performed in parallel, the first comparing growth of C4-2B$_{57}$ cells to parental cells and a second experiment comparing C4-2B$_{57}$ cells to control ribozyme transfectants. At monthly intervals up to 3.5 months, tumor volumes [22] and serum PSA [18] were measured according to the previously published methods.

Results

Pln detection and knockdown clone production

As shown in Figure 1, Pln transcript was readily detected by RT-PCR in three commonly studied prostate cancer cell lines including LNCaP (Panel a), C4-2 (Panel b) and PC3 (Panel c). All three-cell lines expressed roughly similar amounts of transcript encoding Pln. Since C4-2B is considered as a bone homing human prostate cancer LNCaP subline, in all further experiments the C4-2B cell line was chosen as the target which also expressed Pln transcript (Figure 2a) and protein (Figure 1d). A 75–80% reduction of Pln expression was achieved by transfecting a ribozyme specifically targeting Pln into C4-2B cells. Three independent knockdown subclones (C4-2B$_3$, C4-2B$_{11}$ and C4-2B$_{57}$) were selected for further study and to eliminate the possibility of insertional and copy number variation artifacts in the Pln-stably transfected C4-2B subclones. The three subclones were selected from a total of 80 Zeocin resistant clones generated by transfection as those having the lowest detectable levels of Pln mRNA, but meeting the selection criteria by which growth rates in the presence of serum were not affected. In addition to these knockdown clones, several Zeocin-resistant transfected subclones with Pln mRNA expression levels comparable to the parental and untransfected clones also were selected, as were lines in which a ribozyme with scrambled annealing arms (control ribozyme) was transfected. All of these lines were considered to provide useful controls

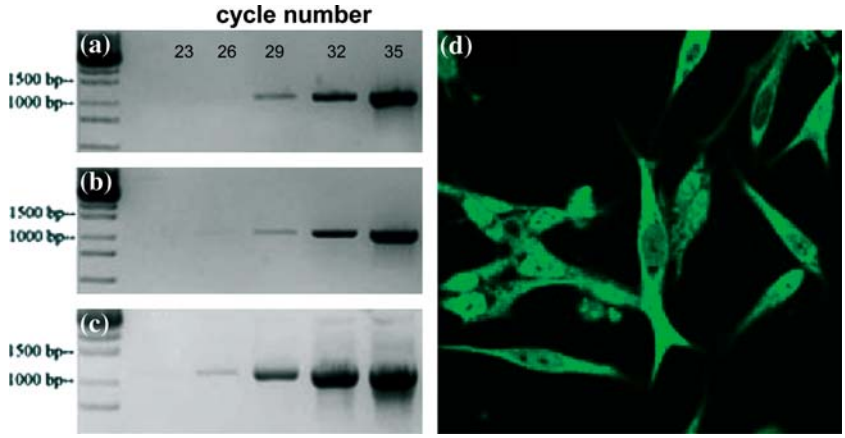

Figure 1. Pln detection by RT-PCR or by immunofluorescence in prostate cancer cell lines. Panel (a) (LNCaP), Panel (b) (C4–2) and Panel (c) (PC3) show the results of RT-PCR amplification of Pln in prostate cancer cells lines with increasing cycle number (23,26,29,32,35 cycles). The amplification region includes an approximately 1100 bp segment comprising most of domain I and a portion of domain II, near the 5′ end of the transcript where the ribozyme targeted sequences are located. Each reaction mixture contained cDNA synthesized from 3.1 μg of total RNA isolated from each cell line. Pln mRNA is abundantly expressed by all three cell lines under normal growth conditions, with slightly more in PC3 cells detectable in the 26 cycle lane. Panel d shows the results of immunofluorescent staining of C4-2B cells with a polyclonal antibody specific for Pln. Pln staining appears uniformly throughout the cells and abundantly in the secretory pathway. Parallel experiments with a non-immune antibody were dark (not shown).

for distinguishing possible effects on cell behavior attributable to the transfection process itself and/or the presence of Zeocin. Figure 2 shows the quantification of Pln transcript in the three knockdown clones (C4-2B$_3$, C4-2B$_{11}$ and C4-2B$_{57}$) based on real time PCR analysis (Figure 2a) and the quantification of the Pln protein release in the medium based on quantitative dot blot hybridization (Figure 2b). This procedure is superior to the Western blot analysis of this protein owing to its large size, charge, and heterogeneity in glycosylation. Based upon a standard curve using purified Pln, along with cell counting, we were able to estimate Pln secretion levels into medium as 7–8 fg/cell/day for C4-2B cells, and between 1–2 fg/cell/day for all knockdown cell lines during growth phase. All three knockdown clones had similar knockdown of transcript and protein; however, the extent of knockdown detected using these procedures was uniformly greater when assessed at the mRNA level by RT-PCR (less than 25% control remaining) than the assessment of protein level (25–35% control remaining.) This was true when protein knockdown was quantitated by dot blot (see above) or estimated by immunofluorescence (data not shown). Non-knockdown ribozyme control plasmid transfected clones had levels of both Pln mRNA and protein comparable to the untreated control clones (data not shown). Throughout the course of these experiments, we observed that ribozymes remained stably expressed and Pln knockdown persisted for at least 10 passages of *in vitro* cell culture. All *in vitro* experiments were performed with subclones during the first 10 passages, after which they were discarded.

Response to growth factors

Figure 3 shows the responses of the three Pln knockdown cell lines to exogenously added growth factors including two HBGFs (FGF-2 and VEGF-A) and a non-HB growth factor, insulin-like growth factor-I (IGF-I). In preliminary experiments, HGF and EGF also were included in the culture medium, but no differences in the responses between parental cells and clones was observed. For parental C4-2B cells, the response to FGF-2 was significantly higher than that to VEGF-A (compare Figures 3a and 3b), but both factors were able to maintain cells in culture over a period of one week. In contrast, cells in medium completely devoid of growth/survival factors showed a progressive loss of adhesiveness, especially after day 4. After 7 days of treatment with the growth factors, all three Pln knockdown subclones showed a significantly lesser proliferative response after treatment with VEGF-A and FGF-2 (Panels 3a, 3b), compared to the parental C4-2B cells. In contrast, the parental and Pln knockdown clones responded equally well to the non-HBGF, IGF-I (Panel 3c), although the C4-2B cell numbers were not as high as in the presence of FGF-2 (compare Figures 3b and c). In addition, proliferation of all these cell lines was similar when grown in medium containing 10% (v/v) FBS when monitored daily over a period of 7 days (data not shown). Statistical analysis showed that, the growth of C4-2B$_3$ was significantly reduced in the presence of VEGF-A ($P < 0.001$) or FGF-2 ($P < 0.05$) treatment by day 7 compared to the parental C4-2B or control ribozyme transfected cells. Conversely, there were no statistically significant differences in growth response to IGF-I after one week. Reduced proliferation in response to VEGF-A and FGF-2 also was observed for C4-2B$_{11}$ and C4-2B$_{57}$ clones at the 7 day point, ($P < 0.05$ in all cases). Again, C4-2B$_{11}$ and C4-2B$_{57}$ clones responded to IGF-I comparably to that of the parental line or non-knockdown controls. Over the first 4 days in culture medium containing 0.2% serum, a period prior to the time when significant detachment began to occur, all

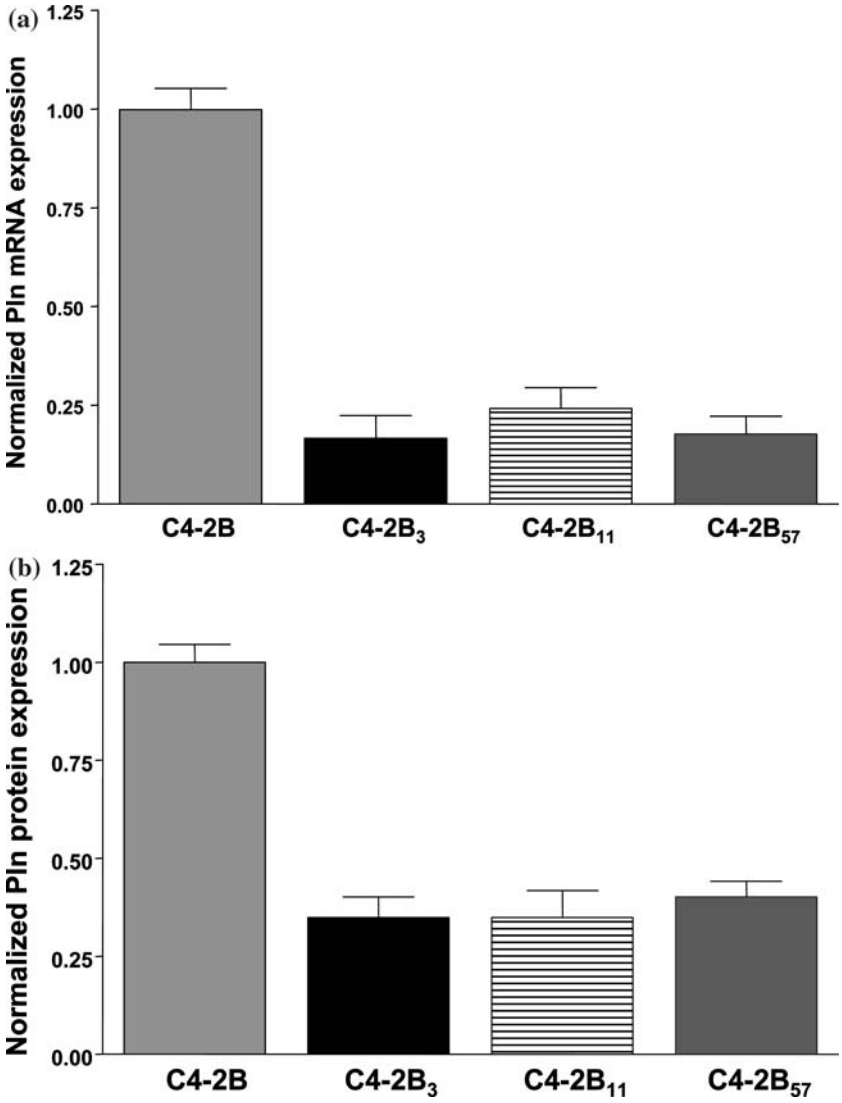

Figure 2. Analysis of Pln knockdown subclones. (a) Expression of Pln mRNA was determined by quantitative real time PCR in C4-2B cells transfected with the Pln ribozyme and normalized to the level of expression in the parental line which was assigned an arbitrary value of 1.0. In all measurements, RNA levels are expressed relative to GAPDH mRNA levels which were used as an internal load standard. (b) Pln protein expression was quantified in medium from the three selected clones by dot blot hybridization. Pure basement membrane Pln was used to construct the standard concentration curve.

four cell lines remained viable and cell number increased similarly. There were no statistically significant differences in cell number between C4-2B and C4-2B$_{3,11,57}$ knockdown clones on day 4 in the absence of serum. Finally, when measured, C4-2B cells were found to make by themselves significant amounts [5 ng/day] of VEGF-A (also see Figure 8), but little or no FGF-2 [<0.5 pg/ml] (data not shown). In summary, we observed a consistent reduction in the ability of all the Pln knockdown subclones to maintain growth in the presence of exogenous HBGFs, but all lines responded similarly to IGF-I or serum growth factors.

We next tested if the loss of growth responses to HBGFs could be rescued by the addition of either Pln-containing conditioned medium from parental C4-2B cells or by addition of a heparan sulfate modified recombinant preparation of the first domain of Pln that we have shown in previous studies [21] to possess the growth factor supporting properties of intact Pln. As shown in Figure 4a, parental C4-2B cells, a control ribozyme transfected clone and two of the knockdown clones, C4-2B$_3$ and C4-2B$_{57}$, grew similarly in early culture in the presence of FGF-2 plus conditioned medium, but, by day 5, growth of the two knockdown clones plateaued whereas the parental line and the control clone continued to grow. This indicated that re-addition of Pln at levels similar to that produced by parental cells was insufficient to rescue the growth response to FGF-2. We next tested if addition of purified domain I of Pln could rescue the day 5 response in the C4-2B$_{57}$ cell line. As shown in Figure 4b, addition of the heparan sulfate bearing Pln construct also failed to rescue growth of the knockdown clone in long term culture in the presence of FGF-2.

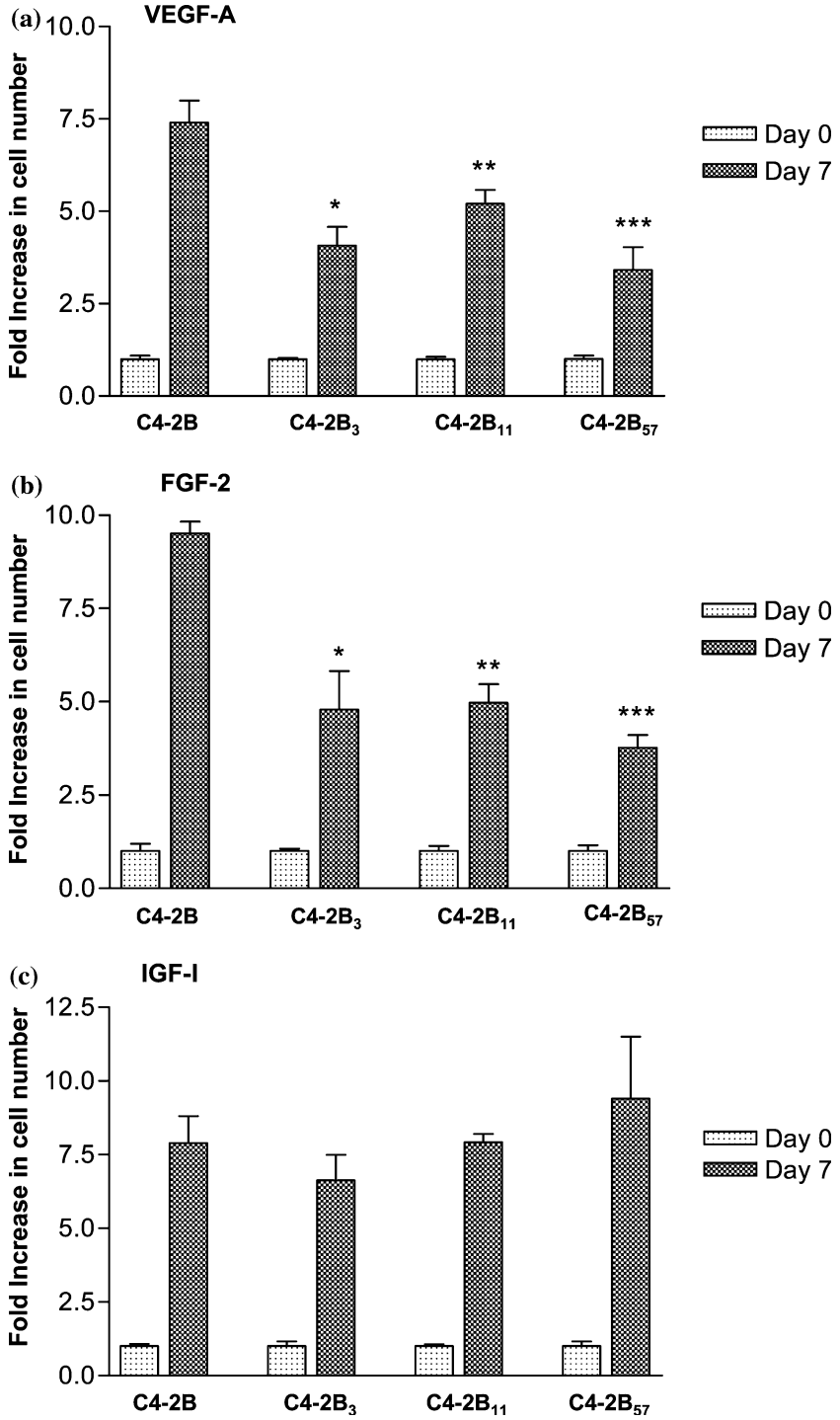

Figure 3. Growth responses to various growth factors by C4-2B and Pln-deficient subclones. Parental and ribozyme knockdown subclones of C4-2B cells were cultured in low glucose DMEM containing 0.2% (v/v) FBS supplemented with 30 ng/ml of VEGF-A [Panel (a), (*$P < 0.003$, **$P < 0.027$, ***$P < 0.001$)] or FGF-2 [Panel (b), (*$P < 0.02$, **$P < 0.0035$. ***$P < 0.003$)]. IGF-I, a non-HBGF, was used as a control under the same conditions (Panel c). Serum depleted (0.2%) medium and medium containing 10% (v/v) FBS also were used as controls and growth was similar in all cell lines (data not shown). VEGF-A and FGF-2 did not stimulate growth of any of the Pln knockdown subclones to the same extent as detected for the parental line, C4-2B. In contrast, IGF-I treatment elicited a similar growth effect for all the cell lines tested.

Soft agar assays

To assess the ability of Pln knockdown to interfere with anchorage independent growth, cells were cultured in soft agar over a two week period. As shown in figure 4, panels a–d, parental C4-2B cells formed uniform round cell clusters that continued to grow throughout the experimental period reaching 150–500 μm by the end of the incubation period. In contrast, all three knockdown lines showed dramatically diminished growth in soft agar, forming irregular, smaller cell clusters that did not grow beyond 60–70 μm. Interestingly, the cell–cell con-

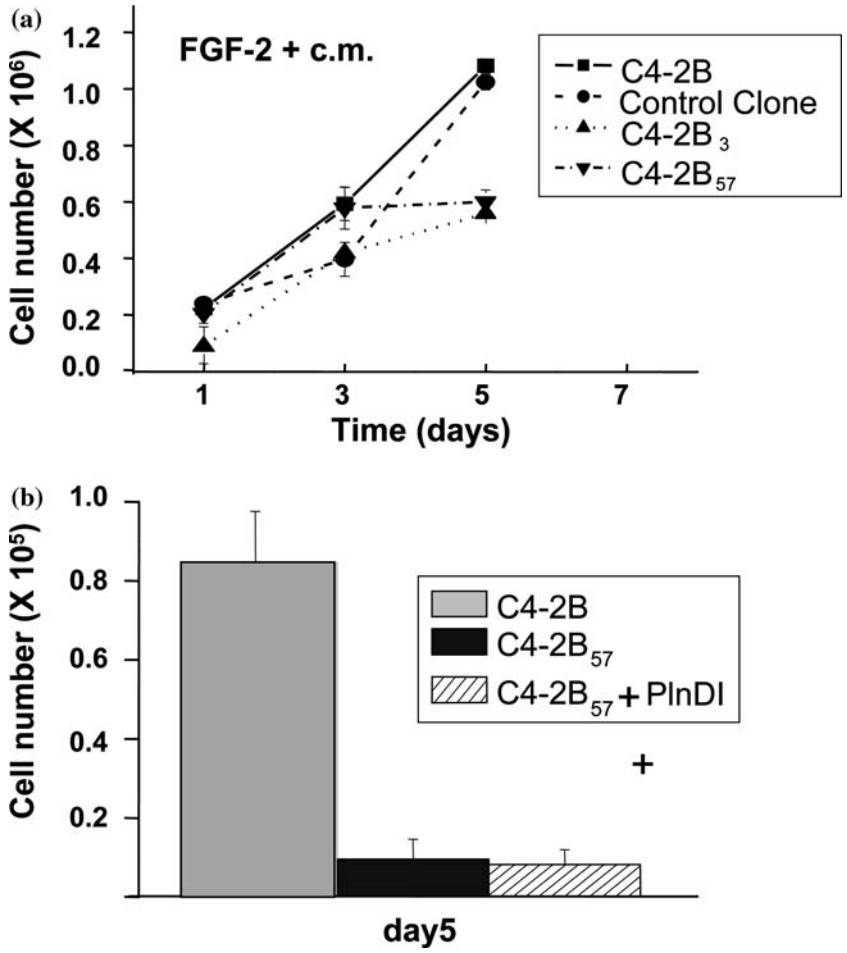

Figure 4. Addition of exogenous Pln does not rescue growth responses to FGF-2 in Pln knockdown C4-2B cells. Panel (a). Conditioned medium (c.m.) from parental C4-2B cells failed to rescue the proliferation of Pln knockdown clones C4-2B$_3$ and C4-2B$_{57}$ cultured in FGF-2 supplemented 0.2% serum medium. Cells (1×10^5) were seeded into 6-well plates and FGF-2 (30 ng/ml) was added to all wells. A control clone transfected with a non-specific ribozyme showed the same growth pattern as the parental C4-2B line over 1 week in culture. Both knockdown clones reached a growth plateau after day 3 even when HBGF and Pln were added back. Panel (b). Purified Pln domain I protein (5 μg/ml) failed to rescue the growth response to FGF-2, whereas parental cells continued to proliferate through the fifth day in culture. Cells (1×10^4) were seeded into 96-well plates in serum depleted medium containing 30 ng/ml FGF-2.

tacts formed by the knockdown clones seemed to be weaker than controls, causing the knockdown cell clusters to appear grape-like rather than round. An assessment of initial colony numbers for all of the cell lines failed to reveal any obvious difference in colony number or ''take'', but rather the lack of Pln appeared to affect colony growth and cell–cell adherence. Despite the altered cellular morphology of the knockdown colonies formed by the subclones, we did not observe clear signs of apoptosis and the cells remained viable for at least 14 days. For further assessment of three-dimensional growth potential in a physiologically relevant condition, one subline, C4-2B$_{57}$ was chosen for growth in SCID mice, as discussed below.

Tumor growth in vivo

Of the three Pln knockdown clones, only C4-2B$_{57}$ proved to be reliably tumorigenic. It was thus chosen to be used for the animal study, and was inoculated into a SCID mouse model as described previously [17]. Two independent animal studies were performed, the first comparing C4-2B$_{57}$ Pln ribozyme knockdown cells with parental cells and the second comparing control ribozyme and active ribozyme transfected cells. In each case similar final results were obtained, although the lag time between tumor cell inoculation and growth of the tumors varied significantly from one tumor to the next, even for the parental C4-2B cells. In both independent studies, the size of the tumors generated and the circulating PSA level detected in the mice was reduced considerably during the twelve week (study one) and fourteen week (study two) data collection period in the Pln knockdown subclone C4-2B$_{57}$ when compared to the parental control or a control ribozyme transfected line. In the case of the representative tumors shown, the size of the tumor induced by C4-2B$_{57}$ cell inoculation was approximately one-fifth that of the tumor formed by the C4-2B control transfected clone (Figure 6a) and the PSA level was detectable only after two months in the mice carrying the C4-2B$_{57}$ subclone (Figure 6b) and similarly in study two (data

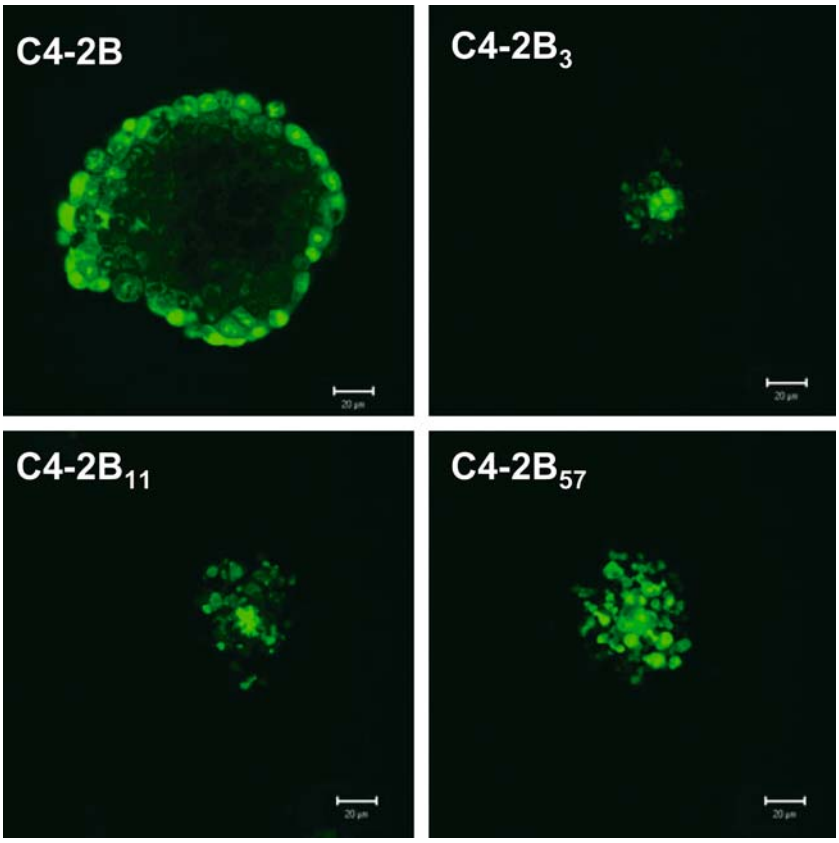

Figure 5. Ribozyme knockdown subclones grow poorly in soft agar. Colony formation in soft agar shows that the parental line C4-2B generates large, round and cohesive colonies, whereas all the knockdown clones lose this characteristic. C4-2B_3, C4-2B_{11} and C4-2B_{57} show not only a reduced colony size, but also a disrupted morphology. Cells are loosely organized and the colony not as regular as the parental line, suggesting that the lack of Pln interferes with the ability to produce normal adhesive ECM.

not shown). Additionally, histological sections of the tumor xenografts dissected from the SCID mice revealed that, in contrast to the tumors formed from cells with normal Pln levels (Figure 7a and c), Pln knockdown cells formed tumors with lower levels of neovascularization (Figure 7b and d, see arrows). The marked difference of neovascular formation between control and Pln ribozyme-transfected prostate tumors was obvious when examined under light microscopy in tissue sections stained with hematoxylin and eosin (H & E, Figure 7 a and b, see arrows). These results were confirmed by the staining of tumor vasculature using a CD31(PECAM-1) antibody prepared and affinity purified from a goat (Figure 7c and d, see arrows). In comparison to control vribozyme or parental cell tumors, Pln knockdown C4-2B_{57} tumors had fewer and smaller vessels, often without red blood cells in the intravascular space suggesting poor blood perfusion. A microvessel density count revealed that the control group ($N = 4$) had 12.3 ± 1.3 (standard error) and the ribozyme group had 7.5 ± 0.3 microvessels/mm square. When CD31 positive total pixilated area was compared using Adobe Photoshop 6.0, the total area was reduced from 29.5% (control) to 12.9% (knockdown). Despite this loss of vasculature, however, the C4-2B_{57} tumors that formed did not appear to be necrotic.

VEGF-A production by C4-2B and Pln knockdown clones

The failure of the knockdown cells to form well vascularized tumors prompted us to examine the production of angiogenic HBGFs by the parental C4-2B cells and their knockdown derivatives C4-2B_3, C4-2B_{11} and C4-2B_{57}. As shown in Figure 8, the parental line produces approximately 5 ng/ml/day of VEGF-A when measured using a commercial standard and an ELISA based assay. Similar assays with a commercial kit showed that these cells made little if any FGF-2 (data not shown). Examination of VEGF-A production by all three Pln knockdown clones showed that the levels of secreted VEGF-A were significantly reduced compared to controls, particularly for sublines C4-2B_3 and C4-2B_{57}, P values ≤ 0.002 for C4-2B_3 and C4-2B_{57}; ≤ 0.02 for C4-2B_{11}.

Discussion

Cell growth and differentiation occur in the context of microenvironments in which HSPGs play major regulatory roles [23] . For prostate cancer cells metastasizing to other tissues such as Pln-rich bone marrow, a network of potential interactions involving both autocrine and paracrine HS-dependent mechanisms exists. In this

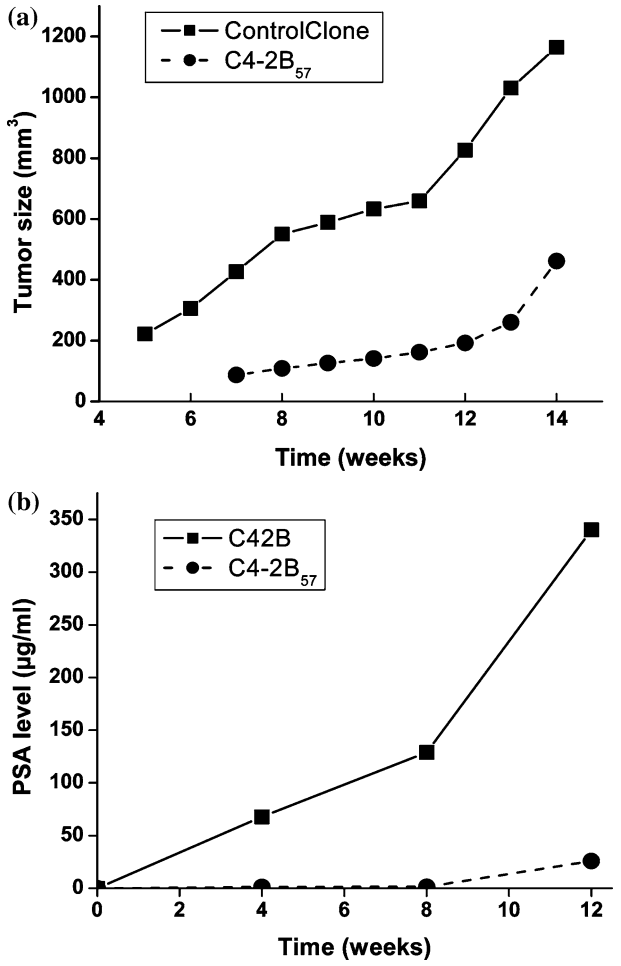

Figure 6. Tumor volume (a) and serum PSA (b) in SCID mice inoculated with the Pln knockdown ($C4\text{-}2B_{57}$) and cell lines expressing normal levels of Pln. Cell lines were introduced into SCID mice as described in Materials and methods. Tumor volume (mm^3) and serum PSA (ng/ml) and were monitored over a 3.5 month period. By both criteria, the cell line containing active Pln ribozyme ($C4\text{-}2B_{57}$) grew small tumors relative to the control ribozyme cell line or the parental line (C4-2B), and PSA remained undetectable over the first eight weeks. The experiment with the knockdown line was repeated twice with similar results. The rebound in the last few weeks is discussed in the text.

study, we demonstrated the relevance of Pln, the major HSPG of basement membranes, endothelial cells and bone stromal ECM, to HBGF-dependent growth responses of prostate cancer cells. For our study, we used a highly metastatic, androgen-independent human prostate cancer cell line C4-2B, generated from parental androgen-dependent LNCaP cells, as a model to illustrate the potential role of Pln in prostate tumor growth both *in vitro* and in SCID mice [24]. This line was chosen for its ability to mimic the natural course of prostate cancer progression and prostate cancer bone metastasis in patients, however, PC-3, parental LNCaP and C4-2 cell lines all expressed Pln mRNA suggesting that the role of this HSPG is not cell line specific (Figure 1).

The utilization of a highly efficient, nuclear-targeted expression system for ribozymes transfected into mammalian cells allowed us to achieve significant knockdown of nascent Pln transcripts in multiple sublines of C4-2B prostate cancer cells. Stably transfected subclones of the C4-2B cell line displayed >75% Pln reduction at the mRNA level. We controlled for a more precise and absolute quantification of the efficiency of Pln knockdown using various approaches, including the evaluation of Pln released in the medium of the subclones, since the protein is secreted into the conditioned medium as an ECM component. We observed a slight difference between the quantification of mRNA and the quantification of the protein secreted in the medium by the stably transfected subclones, with reduction of >50% compared to the untransfected parental C4-2B cell line. This difference may be contributed by Pln synthesis, transport, secretion, degradation and subsequent binding to other extracellular matrix proteins.

The marked reduction of Pln did not affect anchorage-dependent cell proliferation on plastic dishes in the presence of 10% serum or in the first four days of culture in serum depleted medium. Subclones growing on plastic maintained growth rates and doubling times comparable to the parental untransfected cell line. In contrast, anchorage-independent growth was greatly affected, as shown by the dramatically decreased colony growth (smaller colonies) in the soft agar assay (Figure 5). To evaluate the impact of the lack of Pln on the HBGF-driven proliferative responses, we tested the growth responsiveness of the Pln-deficient subclones to two representative heparin binding growth factors that are known to be rich in the bone marrow environment, namely FGF-2 and VEGF-A. The roles of these two growth factors in cancer progression and metastasis previously have been demonstrated in several systems [12, 25–29]. We found that both FGF-2 and VEGF-A were able to maintain C4-2B and knockdown lines in culture even up to one week, with FGF-2 better able to support long term growth and proliferation. The ability of VEGF-A to stimulate modest proliferation was somewhat of a surprise, since this factor is more often associated with angiogenesis. Nonetheless, a recent report also indicated VEGF-A can support autocrine growth stimulation of mammary cancer cells [30]. Pln, a HBGF co-receptor, is known to be a major activator of FGF-2 responsiveness, where it promotes high affinity receptor binding *in vitro* and angiogenesis *in vivo* [31]. Moreover, binding of HBGFs to Pln stabilizes angiogenic growth factors against incorrect folding or proteolysis [32, 33] providing a high capacity reservoir of growth factors for normal and pathogenic cell growth. We thus tested if re-addition of Pln in conditioned medium from the parental cell line or as a purified heparan sulfate-bearing recombinant domain I could rescue responses to FGF-2. The negative results reported in Figure 4 were at first disappointing, however, findings in a recent report [34] demonstrating that tumor-derived Pln rather than host Pln is needed to support epidermal tumor growth may explain these results. At least for Pln knockdown C4-2B cells, co-injection of Matrigel™ during *in vivo* tumor growth studies also failed to rescue the subcutaneous growth of

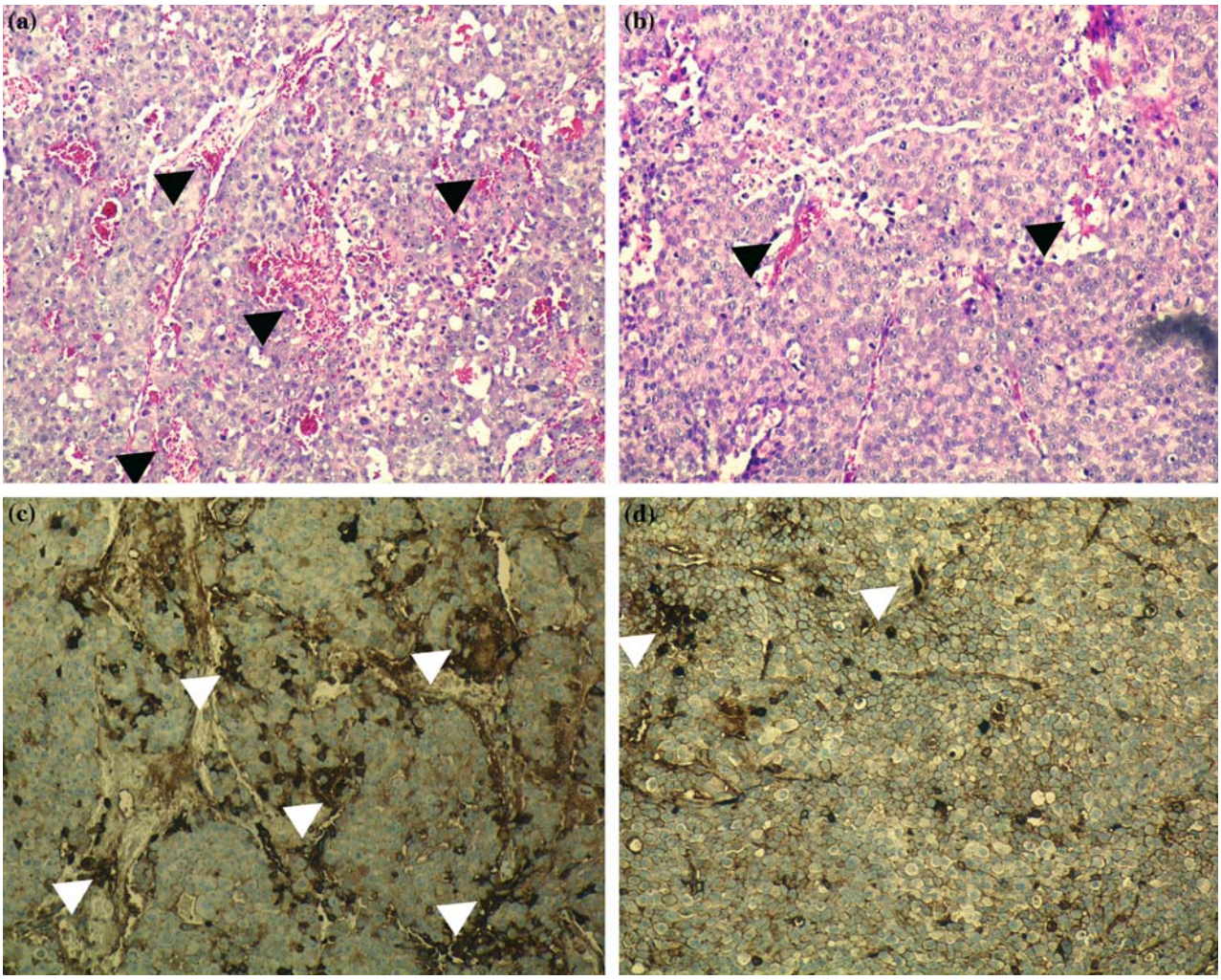

Figure 7. Histological and immunohistochemical assessment of tumor sections obtained from SCID mice inoculated with either control ribozyme, or Pln ribozyme knockdown C4-2B$_{57}$ cells. Abundant blood vessels (arrows) were detected in tumors harvested from animals inoculated with parental C4-2B cells (not shown) or C4-2B cells transfected with a nonspecific ribozyme (Panel a). In contrast, less abundant and much smaller blood vessels were associated with tumors harvested from SCID mice inoculated with C4-2B$_{57}$ cells (Panel b). These results were confirmed by immunohistochemical staining of blood vessels using a CD31 (PECAM-1) antibody (Panels c and d represent the comparative vessel distribution in tumors obtained from the respective control and Pln knockdown clone-inoculated mice). Arrows indicate the locations of blood vessels. Magnification of all pictures is 175×.

the prostate cancer cells. A molecular explanation for these observations could be either that Pln supports tumor growth through an integrated function that requires biosynthesis or alternatively that it must be concentrated at or near the cell surface, a process that might require that it be complexed with other proteins during synthesis. In this light, the results of Marchisone et al. [10] are of interest, since growth of Pln knockdown Kaposi sarcoma cells in nude mice was contextually dependent on the inclusion of Matrigel™ during innoculation. These findings together suggest that the role of Pln in cancer cell behavior is sensitive both to tumor cell origin and to ECM context.

In the present study, we showed that reduction of Pln expression significantly compromised the proliferative responses of prostate cancer cell lines to both FGF-2 and VEGF-A over a 7 day period *in vitro* (Figure 3). In contrast, responses to IGF-I or serum were unaffected over this same time frame. The lack of cohesiveness of the cells in the colonies growing in soft agar also was of interest. Pln contains, in addition to the growth factor function associated with the heparan sulfate chains in domain I, four additional domains that interact with a wide number of matrix molecules and surface receptors [reviewed recently in Farach-Carson et al, 2004 [35]. Experiments are ongoing in our laboratory to further identify the domains of Pln that may be responsible for the loss of cell–cell adhesion that we observed.

It has been hypothesized that Pln deposition in the metastasizing tumor functions as a scaffold for new capillary formation during growth and development of neovasculature [36]. The *in vivo* significance of Pln in supporting prostate tumor formation was confirmed in two studies that we performed in SCID mice (Figures 6 and 7). Of the knockdown clones, only C4-2B$_{57}$, which also showed the best growth in soft agar (Figure 5) produced tumors consistently even when Matrigel™ was added during tumor inoculation. Interestingly, not only

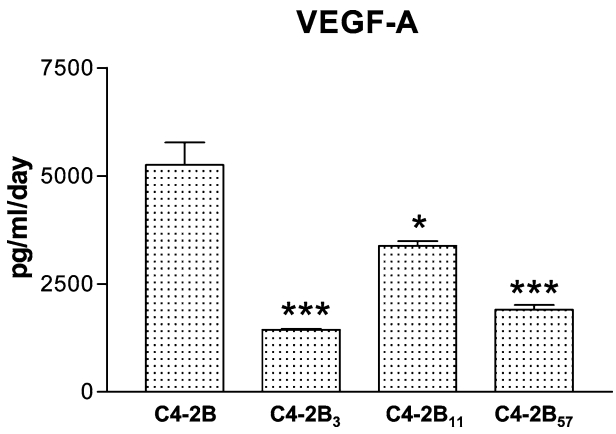

Figure 8. C4-2B Pln knockdown cell lines make reduced amounts of VEGF-A. The C4-2B cell line produces significant VEGF-A but little or no FGF-2 (see text). Cells from each line were grown to 70% confluency in a T75 flask, then switched to serum depleted (0.2%) medium for 48 h. The levels of VEGF-A in cleared medium from parental and knockdown lines were determined using commercially available ELISA assay (R&D Systems). As shown, all three Pln knockdown lines produced significantly less VEGF-A than did control C4-2B cells [$P < 0.002$ for C4-2B$_3$ and C4-2B$_{57}$ (***) and < 0.02 for C4-2B$_{11}$ (*)].

were the tumors generated by the Pln ribozyme-stably transfected subclone C4-2B$_{57}$ considerably smaller in size than the control C4-2B tumors or tumors formed from C4-2B cells transfected with an inactive control ribozyme, but also the PSA levels in the serum of animals bearing tumors derived from the Pln deficient subclone were dramatically reduced, especially during the first eight weeks when they were undetectable. After three months, some rebound in PSA production was seen (data not shown) but the most likely explanation for this is loss of the ribozyme function *in vivo* after this long time period. Finally, these observations are supported by the histological and immunohistochemical detection of blood vessels within the tumors where, as might have been expected, much reduced number and smaller blood vessels were detected in the formalin and paraffin-fixed specimens from the knockdown tumors than those formed from parental C4-2B cells or control transfectants (29.5% vs. 12.9% in pixel analysis of CD31 positive immunostained area in Figure 7c, d). Our findings are consistent with the recent suggestion, based on reduced tumor growth in Pln heparan sulfate deficient mice [37], that Pln plays an essential role in tumor growth and its failure to deliver FGF-2 might result in defective angiogenesis. An additional reason that Pln deficiency could reduce tumor angiogenesis arises from the observation that Pln knockdown also appears to lower the production of VEGF-A by tumor cells. Interestingly, a microarray analysis that we recently conducted comparing gene expression in C4-2B parental cells, a control ribozyme transfectant, and two knockdown clones (C4-2B$_3$ and C4-2B$_{57}$) revealed that among all the HBGFs, their common receptors, and the HSPGs represented on the Affimetrix array, only PDGF was reduced significantly in addition to VEGF-A. This was confirmed by RT-PCR (data not shown). It is intriguing to speculate that Pln biosynthesis supports autocrine cellular signaling pathways that maintain gene expression of angiogenic growth factors such as VEGF-A and PDGF, and that this explains, at least in part, the poor growth and vascularization of the knockdown clones at

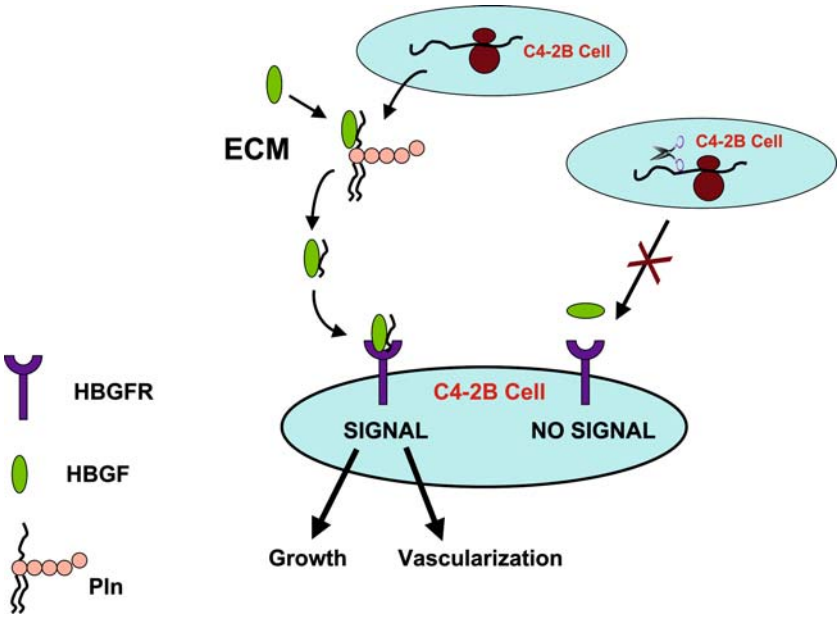

Figure 9. Model for Pln function in C4-2B prostate cancer cell growth and vascularization. Pln is secreted by C4-2B cells where it binds HBGFs (VEGF-A or FGF-2) present in the matrix either secreted by tumor or surrounding stroma. The co-receptor function allows delivery of active HBGFs bound to heparan sulfate to growth factor receptors present on cancer cells. Signals resulting from receptor activation support growth and angiogenesis. Ribozyme (scissors)-mediated knockdown of Pln production by the C4-2B cells reduces the co-receptor function and abrogates signaling required for tumor growth and vascularization. This suggests that Pln biosynthesis by the cancer cells is an essential component of prostatic tumorigenesis.

subcutaneous sites in SCID mice. Further studies utilizing larger numbers of mice and tumor cells inoculated at multiple sites of potential metastasis with and without Matrigel™ are warranted to systematically investigate the mechanisms underlying the link between Pln biosynthesis and angiogenesis. Taken all together, these collective studies demonstrate the essential role of Pln in HBGF-dependent growth under a variety of *in vitro* conditions and in tumor growth and vascularization in a mouse model of prostate cancer reminiscent of the human disease. A model illustrating this concept is shown in Figure 9. Interesting, recent data indicate that a likely pathway through which this could occur is the Sonic Hedgehog pathway [38]. In this context, Pln or the pathways it activates could be an attractive potential therapeutic target. By abrogating Pln expression in prostate cancer cells, HBGF signaling in prostate cancer cells may be eliminated with concomitant loss of tumor growth and angiogenesis.

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